Acetyl-L-carnitine restores synaptic transmission and enhances the inducibility of stable LTP after oxygen-glucose deprivation

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Abstract

Hypoxic circumstances result in functional and structural impairments of the brain. Oxygen-glucose deprivation (OGD) on hippocampal slices is a technique widely used to investigate the consequences of ischemic stroke and the potential neuroprotective effects of different drugs. Acetyl-L-carnitine (ALC) is a naturally occurring substance in the body, and it can therefore be administered safely even in relatively high doses. In previous experiments, ALC pretreatment proved to be effective against global hypoperfusion. In the present study, we investigated whether ALC can be protective in an OGD model. We are not aware of any earlier study in which the long-term potentiation (LTP) function on hippocampal slices was measured after OGD. Therefore, we set out to determine whether an effective ALC concentration has an effect on synaptic plasticity after OGD in the hippocampal CA1 subfield. A further aim was to investigate the mechanism underlying the protective effect of this compound. The experiments revealed that ALC is neuroprotective against OGD in a dose-dependent manner, which is manifested not only in the regeneration of the impaired synaptic transmission after the OGD, but also in the inducibility and stability of the LTP. In the case of the most effective concentration of ALC (500 μM), use of a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) revealed that the PI3K/Akt signaling pathway has a key role in the restoration of the synaptic transmission and plasticity reached by ALC treatment.

Keywords: oxygen-glucose deprivation, acetyl-L-carnitine, long-term potentiation, PI3K/Akt, neuroprotection, ischemia

1. Introduction

Cerebral ischemia results in failure of the bioenergetic processes. The key element of this phenomenon is the breakdown of the mitochondria, which leads to the failure of ATP production and the excessive release of Ca^{2+}. It causes not only excitotoxicity, but also the generation of reactive oxygen species, the release of proapoptotic signal proteins and the subsequent neuronal death (Ten and Starkov 2012). It has been well established that the hippocampus, and especially the CA1 subfield, is extremely sensitive to hypoxic-hypoglycemic conditions (Kirino 1982, Pulsinelli et al. 1982), which can occur, for example, during ischemic stroke or cardiac arrest. Hippocampal slices are widely used to investigate the injury induced by ischemic events and to measure the effects of different pharmacological interventions against the neuronal damage (Picconi et al. 2006, Molz et al. 2015). Oxygen-glucose deprivation (OGD) mimicking brain ischemia can result in the reversible or irreversible depression of neurotransmission, depending on the duration of this insult. Electrophysiological recordings from slice preparations allow continuous monitoring of the changes in the evoked electrical responses which can occur as a result of the ischemic event or potential neuroprotective agents (Picconi et al. 2006, Nistico et al. 2008). Furthermore, the hippocampus is a suitable and generally used model system for the study of synaptic plasticity, and especially long-term potentiation (LTP), since it is involved in learning and memory (Ho et al. 2011). As a result of the vulnerability to hypoxic-hypoglycemic circumstances of this structure, the effects of ischemia are manifested in functional and morphological damages, e.g. LTP impairment and the loss of dendritic spines (Kocsis et al. 2014), or in irreversible harmful processess ending in cell death.

Acetyl-L-carnitine (ALC) is an endogenous compound, which is a short-chain acetyl ester of L-carnitine (Bremer 1983, Bieber 1988) synthesized in the human brain, liver and kidney by the enzyme ALC transferase. This molecule affects the overall energy metabolism and cell functions, including the regulation of the lipid, carbohydrate and protein metabolism (Rapoport 1999), it boosts mitochondrial ATP production and it protects the mitochondria against oxidative stress (Zanelli et al. 2005). ALC is actively transported across the blood-brain barrier to the brain (Burlina et al. 1989), where it has a relative high concentration (Shug et al. 1982). Several studies have demonstrated the neuroprotective effect of ALC against different neurodegenerative diseases, such as Alzheimer's disease, ischemia or neuropathic pain (Di Cesare Mannelli et al. 2009, Zhou et al. 2011, Xu et al. 2015). We previously

investigated the neuroprotective effect of ALC against global hypoperfusion in a 2-vessel occlusion (2VO) model (Kocsis et al. 2014, Kocsis et al. 2015). The effects of ALC were also tested on striatal slices in an *in vitro* ischemia model, where it prevented the loss of the recorded field excitatory postynaptic potentials (fEPSPs) through the activation of M2 muscarinic receptors and the choline uptake system (Picconi et al. 2006). Moreover, the mechanisms underlying the neuroprotective effect of ALC exhibit a great deal of variety, since it provides a substrate reservoir for cellular energy production, facilitates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, enhances different synthesis processes (Fiskum 2004, Di Cesare Mannelli et al. 2010), has antioxidant and anti-apoptotic properties (Zanelli et al., 2005), and induces nerve regeneration by increasing the production and binding of the nerve growth factor (Foreman et al. 1995).

In the present study, our aim was to examine the potential neuroprotective effect of ALC against *in vitro* global ischemia delivered to hippocampal slices. To the best of our knowledge, there has not been any study so far in which the LTP function was measured on a hippocampal slice after OGD. We therefore set out to determine whether an effective ALC concentration has an effect on synaptic plasticity after OGD in the hippocampal CA1 subfield.

2. Experimental procedures

2.1 Animals and housing conditions

Male Wistar rats weighing 200-250 g (N = 21) supplied by Charles River Laboratories, were kept under constant environmental conditions (23 °C; humidity 55 ± 5%; a 12-h/12-h light/dark cycle) and were housed individually in standard plastic cages, where they had free access to food and water. Every effort was made to minimize the number of animals used and their suffering. The principles of animal care (NIH publication No. 85-23), and the protocol for animal care approved both by the Hungarian Health Committee (1998) and by the European Communities Council Directive (2010/63/EU) were followed. Before the experimental procedures, all the rats were in normal health and had no neurogical deficits.

2.2. *In vitro* slice preparation

The preparation and maintenance of rat hippocampal slices were described previously (Kocsis et al., 2014; Kocsis et al., 2015). Briefly, the animals were decapitated and the middle parts of the hippocampi were placed in ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mM): 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$ and 10 D-glucose (all from Sigma, Germany), saturated with 95% O_2 + 5% CO_2. Coronal hippocampal slices (350 μm) were prepared with a vibratome (Leica VT1200S, Germany). Slices were transferred to a holding chamber and allowed to recover in the solution used for recording (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$) for at least 1 h.

2.3. Electrophysiological recordings

For the electrophysiological experiments, slices were transferred to a Haas recording chamber, where the flow rate of the recording solution (34 °C) was 1.5–2 $ml \times min^{-1}$. A bipolar concentric stainless steel electrode (Neuronelektrod Ltd, Hungary) was placed in the stratum radiatum between the CA1 and CA2 regions of the hippocampal slices. The stimulus intensity was adjusted to between 30 and 60 μA (constant current, 0.2-ms pulses delivered at 0.05 Hz) to evoke the half-maximum response. fEPSPs were recorded with a 1.5-2.5 MΩ resistance glass micropipette filled with aCSF. The recordings were amplified with a neutralized, high input-impedance preamplifier and filtered (1 Hz-3 kHz). The fEPSPs were digitized (AIF-03, Experimetria Ltd. Hungary), acquired at a sampling rate of 10 kHz, saved to a PC and analyzed off-line with Origin Pro 8 software (OriginLab Corporation,

Northampton, MA, USA). The fEPSPs were monitored until the amplitudes were generally stable, and a 10-min-long baseline was then recorded, which was followed by a 15-min OGD. The fEPSPs were allowed to recover for 40 min after the OGD, and at the end of this period theta burst stimulation (TBS) was applied (bursts of 4 impulses at 100 Hz with a burst interval of 350 ms) for LTP measurements. After the TBS, changes in fEPSP amplitudes were recorded for a further 35 min (Fig. 1).

2.4. *In vitro* ischemia

In vitro ischemia was delivered by switching for 15 min to an OGD aCSF solution in which sucrose replaced glucose, and gassed with 95% N_2 + 5% CO_2. After the OGD, the slices were perfused with normal aCSF again until the end of the measurements. In the preliminary experiments, OGD was delivered for different periods (5, 8, 12, 15, 16, and 17 min) in order to determine the appropriate length of the ischemia for our study. After shorter terms of OGD, the fEPSPs returned, but the aim was to find the limit when the fEPSPs no longer displayed recovery. Electrical noise could not be excluded completely during the electrophysiological measurements, and it also was present after the elimination of the fEPSPs. This is the reason why the recorded amplitudes never reached the value of zero. Both fEPSP amplitudes and initial slopes were recorded and quantified in all of the measurements; however as no appreciable differences between these two parameters were observed, only the amplitudes are expressed in the figures.

2.5. Application of ALC

ALC (Sigma, Germany) was applied for 25 min at the desired final concentration (125, 250 or 500 μM) in aCSF or OGD aCSF for the control (10 min) and the OGD period (15 min), respectively. In the preliminary studies, ALC was tested in all of the concentrations utilized in the further experiments (25-min wash-in), and none of them influenced the amplitudes (Fig. 3). Nevertheless, all the recordings also were self-controlled, since after the stabilization of the fEPSPs, the recordings started with a 10-min period when the slices were still perfused with normal aCSF solution. This was followed by an additional 10 min, when the aCSF contained ALC. Since no differences were still observed, this ALC wash-in period was regarded as the control baseline during the data analysis. The wash-in of the ALC was

continued during the 15-min OGD, and it was then followed by the recovery period when normal aCSF solution was perfused onto the slices (Fig. 1).

2.6. Application of LY294002

In order to investigate the mechanisms underlying the protective effect of ALC, a phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (Tocris, United Kingdom) was utilized, in a final concentration of 50 μM. It was dissolved in aCSF or OGD aCSF containing 500 μM ALC, and the solution was washed onto the slices during the control and the OGD period, respectively.

2.7. Statistical analysis

In OGD measurements, the fEPSP amplitudes were expressed as a percentage of the 10-min baseline value before OGD, while to express the potentiation after LTP induction the last 10 min of the recovery period following the OGD was regarded as a baseline.

For data presented as means ± S.E.M., statistical analysis was performed with the use of the non-parametric Wilcoxon test and the Mann-Whitney U test. The significance level was established at $p < 0.05$.

3. Results

In the first part of the experiments, our aim was to determine the accurate duration of OGD sufficient for the elimination of the fEPSPs. After shorter OGD periods (5 min or 8 min), the amplitudes displayed a complete recovery. In the case of the 5-min OGD, the fEPSPs demonstrated not only regeneration after the ischemia, but also facilitation (presumably post-ischemic LTP). The increased amplitudes were stable until the end of the recording period. A 12-min period of ischemia was likewise not enough to abolish the fEPSPs, but 16 or 17-min periods of OGD resulted in complete elimination of the amplitudes (Fig. 2). A 15-min period of OGD ($N = 9$) was the shortest which abolished the fEPSPs, and the following experiments were carried out with this model. A slight increase of the fEPSPs was detected immediately after the OGD in almost all measurements of the different experimental groups, and the amplitudes started to decrease only thereafter. This phenomenon is probably due to an increased sensitivity of the potsynaptic NMDA receptors due to the removal of Mg^{2+} ions from the ionophore part during an ischemic event, an excitotoxic circumstance that may have

resulted first in an abnormal facilitation of the synaptic transmission in the CA1 region, and then by the progression of OGD lead to the complete elimination of the fEPSPs.

The potential neuroprotective effect of ALC against 15-min OGD was tested in different concentrations. Based on our preliminary studies, we chose 125, 250, and 500 μM ALC for the OGD measurements. Before these experiments, the potential neuromodulatory effect of ALC was tested on control slices. None of the applied concentrations influenced the fEPSPs (Fig. 3).

125 μM ALC was ineffective in half of the group (N = 6), while in the other half of the experiments (N = 6) it resulted in a slight recovery. Increase of the fEPSPs after OGD was slow and reached its maximum early. The average of the amplitudes in the last 10 min of the recordings was significantly lower (58.91 ± 3.70%) as compared with the control level. As a result of 250 μM ALC (N = 12), the fEPSPs increased continuously and reached the control level within 25 min. In the last 10 min of the recording period, the amplitudes stabilized at a significantly higher level (107.59 ± 2.37%) relative to the control. 500 μM ALC (N = 12) resulted in an even faster recovery of the fEPSPs. There was no significant difference between the values for the last 10 min of the 250 and 500 μM ALC groups, but both were significantly higher than that for the 125 μM ALC group (Fig. 4).

After the 40-min recovery period, LTP was induced by TBS and the fEPSP amplitudes were measured for an additional 35 min. The level of the LTPs was normalized to the last 10 min of the recovery period. The LTP inducibility was tested in the 15-min OGD group, but because of the irreversible elimination of the fEPSPs, no potentiation was observed. The illustration of these results in Fig. 5 was therefore not adequate. From the 6 samples showing partial restoration in the 125 μM ALC group, we observed LTP in only 2 cases. The fEPSPs showed a weak potentiation in the first 10 min after TBS (116.87 ± 1.62%), then started to decrease and at the end of the recordings they had fallen back to the control level (102.79 ± 0.96%). There was no significant difference between the control period and the last 10 min of the recording. 250 μM ALC (N = 12) resulted in a higher potentiation, although the amplitudes showed a slight decrease for 15 min after TBS and stabilized only thereafter (123.96 ± 1.97%). This potentiation was significantly higher as compared with the values for the 125 μM ALC group. 500 μM (N = 12) was the most effective ALC concentration since it resulted not only in complete restoration of the fEPSPs after OGD, but also in a stable LTP. As a

result of the TBS, the amplitudes increased, stabilized immediately and remained stable until the end of the recording period (141.12 ± 0.90%). This LTP is similar to that of tested in control conditions (140.75 ± 0.40%) on hippocampal slices (N = 8). In the 500 μM ALC-treated group, the average of the fEPSP amplitudes in the last 10 minutes of the recordings was significantly higher relative to the control level and to the values of the last 10 min of the 125 and 250 μM ALC groups (Fig. 5).

LY294002, a PI3K inhibitor, was used to reveal the mechanisms underlying the protective effect of ALC, which was manifested in the recovery of the fEPSPs and a stable LTP after OGD. As a result of 500 μM ALC, the amplitudes started to decrease later during the ischemia as compared with the OGD group, but this effect was abolished in the presence of the inhibitor (N = 8). After the OGD period, the fEPSPs did not recover when ALC was applied with LY294002 (Fig. 6). LTP inducibility was also tested in the group that received 500 μM ALC + LY294002, but the complete and irreversible elimination of the fEPSPs resulted in the same results as recorded in the 15-min OGD group. Fig. 7 shows representative traces from the different experimental groups. There were no pathological responses to the stimulation none of the cases, even after the TBS.

4. Discussion

Hypoxic conditions, reperfusion and reoxygenization are central elements of many disorders in the nervous system, e.g. ischemic stroke, traumatic brain injury and heart attack (Ronaldson and Davis 2013). During an ischemic event, the limited blood flow in different parts or in the whole brain results in a complex cascade of molecular processes, which leads to cell death (Thompson and Ronaldson 2014). There is still no effective solution with which to prevent the structural and functional impairments of stroke patients, and it is therefore very important to search for alternative treatments which could rescue the nerve tissue. ALC is a naturally occurring substance which has been demonstrated to be neuroprotective in different diseases (Malaguarnera 2012). ALC is widely distributed in the tissues; its synthesis occurs in the brain, intestine, liver and kidney (Pettegrew et al. 2000). The therapeutic application of this compound is promising since it readily crosses the blood-brain barrier, affects many bioenergetic processes, exhibits antioxidant, antiapoptotic and neuromodulatory effects and enhances nerve regeneration (Jones et al. 2010). Among the numerous mechanisms

underlying the neuroprotective action of ALC, it is likely that this nutrient can serve as an alternative source of acetyl-CoA during or after cerebral ischemia. ALC enhances the aerobic energy metabolism impaired by the breakdown of the pyruvate dehydrogenase complex under hypoxic conditions (Zanelli et al. 2005).

In previous experiments, we investigated the potential neuroprotective action of ALC against global hypoperfusion in a 2VO model of rats. We found that ALC was effective only when it was administered before the ischemic insult, but not as post-treatment (Kocsis et al., 2014). These findings are in accordance with the results of other research groups. In the present study, we examined the effect of this compound against *in vitro* global ischemia on hippocampal brain slices with electrophysiological techniques. Our aim was not only to find the effective concentration of ALC which restores the synaptic transmisson after OGD, but also to measure the LTP function after the recovery period.

After determination of the accurate period of OGD sufficient for the elimination of the fEPSPs, we tested ALC in different concentrations as pretreatment. In preliminary studies, ALC applied after OGD did not have protective effect, which was in accordance with a previous report (Picconi et al., 2006). Hence, the compound was washed into the slices before and during the OGD period.

Though 15-min OGD resulted in the complete elimination of fEPSPs, which was not followed by recovery, there might be differences between each slice as regards the extent of the damage or the number of the potentially salvageable neurons, which may underlie the results revealed in the 125 μM ALC-treated group. Indeed, this concentration was high enough in some cases to achieve a detectable slight neuroprotective effect, probably corresponding to slices with a higher number of salvageable neurons remained following the 15-min OGD. The ineffectiveness of 125 μM ALC in the other half of the experiments suggests that this concentration may be in the edge of the least effective concentration of ALC in this model.

The damages caused by OGD to the neuronal network of the hippocampal CA1 subfield could be partially restored with 125 μM ALC only at the level of fEPSPs, but not at that of a complex process such as LTP. Nevertheless, in 2 cases of the 125 μM ALC group, we detected a slight and decaying potentiation. These results, similarly to the partial restoration of the fEPSPs, may also be accounted for the potential differences in the status of the particular slices exposed to ischemia.

250 and 500 μM ALC displayed similar protective effects in the recovery period, but we found differences between the LTP results of these groups. Despite the complete recovery in the 250 μM ALC group, the pontentiation of the fEPSP amplitudes was not stable after TBS. 500 μM ALC exerted its neuroprotective effect via the restoration of the fEPSPs and also preservation of the synaptic integrity and plasticity.

The PI3K/protein kinase B (Akt) pathway is important during the development of the central nervous system and plays a critical role in mediating survival signals in a wide range of circumstances (Brunet et al. 2001). Furthermore, it has been well established that this signaling pathway mediates synaptic plasticity and memory (Horwood et al. 2006), and is also involved in the cognitive impairment caused by chronic cerebral hypoperfusion (Shu et al. 2013).

Among several mechanisms through which ALC exerts its protective action, the importance of the PI3K/Akt pathway has already been demonstrated. In cortical neuronal cell cultures exposed to oxidative stress-induced neurodegeneration, ALC and α-lipoic acid pretreatment resulted in activation of the PI3K, PKG and ERK1/2 pathways, which have essential roles in cell survival (Abdul and Butterfield 2007). In the present study, we explored the hypothesis that ALC exerts its neuroprotective effect via the PI3K/Akt pathway, which is manifested not only in the recovery of the fEPSPs after OGD, but also in the potentiation of the amplitudes after TBS. Despite the complete recovery and stable LTP reached following 500 μM ALC treatment, LY294002 administration abolished this protective effect.

These results suggest that the PI3K/Akt pathway is necessary for the neuroprotection induced by ALC in different ways. This ALC-activated signaling pathway promotes cell survival mechanisms through which the synaptic transmission recovers after OGD. Additionally, activation of the PI3K/Akt pathway contributes to the enhancement of the synaptic plasticity revealed by the LTP measurements. Nevertheless, there are many other mechanisms connected with the neuroprotective effect of ALC, which can simultaneously take part in the results revealed in our experiments.

Among the various beneficial effects of ALC, its natural presence in the body underlies the feasibility of this compound in patients with high safety. Experiments measuring the potential protective effect of ALC in different animal models of diseases are promising and provide a broad range of information for clinical research. Clinical trials with ALC in patients have so

far rather focused on neurodegenerative diseases (e.g. Alzheimer's disease); however, the numerous studies revealing its neuroprotective effects in ischemic models emphasize the importance of the assessment of the neuroprotective efficacy of ALC also in clinical trials of stroke as well in the future.

5. Conclusions

In this study, we demonstrated that ALC can exert its protective effect on a complex process such as LTP, a phenomenon underlying the basis of learning and memory, which provides further relevance of its therapeutic application. Overall, these results also contribute to the better understanding of the relationship between ALC and the neuroprotection following ischemia.

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7. Conflict of interest

The authors declare that they have no conflict of interest.

8. Legends to Figures

Fig. 1

Schematic timeline of the experimental protocol. ALC was washed onto the slices during the 10-min control and the 15-min OGD period. At the end of the recovery period, LTP was induced by TBS. The last 10 min of the recovery period (symbolized by the gray part of the black line) was regarded as the control baseline of the LTP period.

Fig. 2

Results of the preliminary experiments conducted to determine the sufficient period of OGD. 5-min OGD resulted in complete recovery of the fEPSPs ($N = 2$). In some cases, a noteworthy facilitation was observed after the 5-min OGD ($N = 2$), which was presumably a post-ischemic LTP. The 8- and 12-min OGD were still not enough to eliminate the fEPSPs; these periods resulted in complete or partial regeneration, respectively ($N = 2$ and 4, respectively). Longer OGD periods (16 min ($N = 2$) and 17 min ($N = 2$)) completely abolished the fEPSPs. Data points are means ± S.E.M. of the normalized amplitudes of the fEPSPs.

Fig. 3

The potential neuromodulatory effect of 125 ($N = 6$), 250 ($N = 6$), and 500 μM ALC ($N = 6$) was tested on control hippocampal slices (25-min-long ALC application). None of the concentrations influenced the fEPSPs. Data points are means ± S.E.M. of the normalized amplitudes of the fEPSPs.

Fig. 4

ALC was neuroprotective against 15-min OGD in a dose-dependent manner. The 15-min OGD ($N = 9$) was the shortest period which was not followed by any recovery, and ALC was therefore tested in this model. 125 μM ALC resulted in a slight recovery in only half of the group ($N = 6$), but in the other half ($N = 6$) it was ineffective. 250 and 500 μM ALC ($N = 12$ each) were protective: the fEPSPs recovered completely after the OGD. Data points are

means ± S.E.M. of the normalized amplitudes of the fEPSPs. Asterisks denote significant differences between the control and the last 10 min of the recovery period (**p < 0.01, ***p < 0.001, Wilcoxon test), # denotes significant differences between the last 10 min of the recovery period of each experimental group (### p < 0.001, n.s.: not significant; Mann-Whitney U test).

Fig. 5

The effect of ALC on LTP inducibility. Despite the slight regeneration of the fESPSs in the 125 μM ALC group, LTP could be induced in only 2 cases. The potentiation was weak, and the amplitudes started to decrease immediately, and reached the control baseline at the end of the recordings. 250 μM ALC (*N* = 12) resulted in a significantly higher LTP, but showed a slight decrease for 15 min after TBS and stabilized only thereafter. 500 μM ALC (*N* = 12) was the most effective concentration. It was manifested in significant potentiation, which was stable over time. This LTP is similar to that of tested on hippocampal slices (*N* = 8) in control conditions (inset in the upper right quadrant). Data points are means ± S.E.M. of the normalized amplitudes of the fEPSPs. Asterisks denote significant differences between the control and the last 10 min of the LTP period (***p < 0.001, Wilcoxon test); # denotes significant differences between the last 10 min of the LTP period of each experimental group (### p < 0.001, n.s.: not significant; Mann-Whitney U test).

Fig. 6

The PI3K/Akt pathway has a key role in the neuroprotective effect of ALC. The 15-min OGD (*N* = 9) completely eliminated the fEPSPs, which was blocked by 500 μM ALC (*N* = 12) treatment, but in the presence of LY294002 (*N* = 8) this protective effect of ALC was abolished. Data points are means ± S.E.M. of the normalized amplitudes of the fEPSPs.

Fig. 7

Representative traces of fEPSPs from the different experimental groups recorded in the control, the last 10 min of the recovery, and the last 10 min of the post-TBS period. 125 μM

ALC was effective against the ischemic insult in half of the experiments ($N = 6$), but only 2 of them showed a slight potentiation after TBS. This LTP was unstable and the amplitudes decreased to the pre-TBS level at the end of the recordings (3rd row). Both 250 and 500 μM of ALC resulted in a complete recovery of the fEPSPs; however, stable LTP could only be recorded in the 500 μM ALC-treated group. This effect of ALC was abolished in the presence of LY294002.

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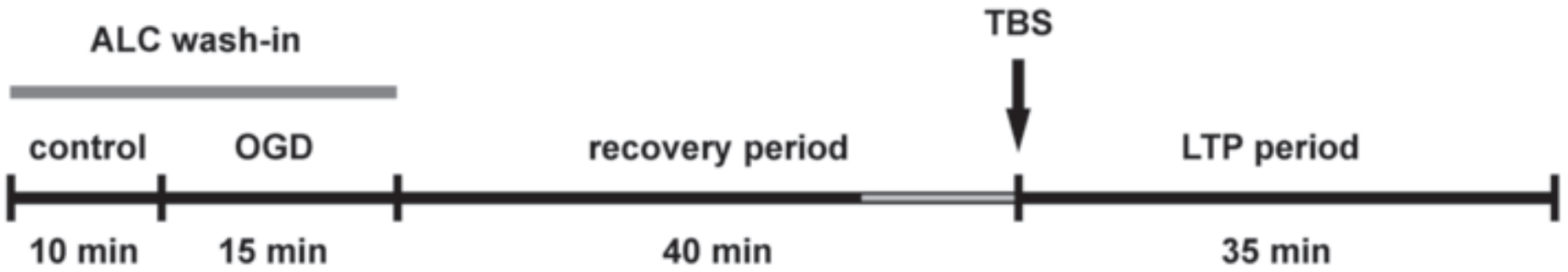

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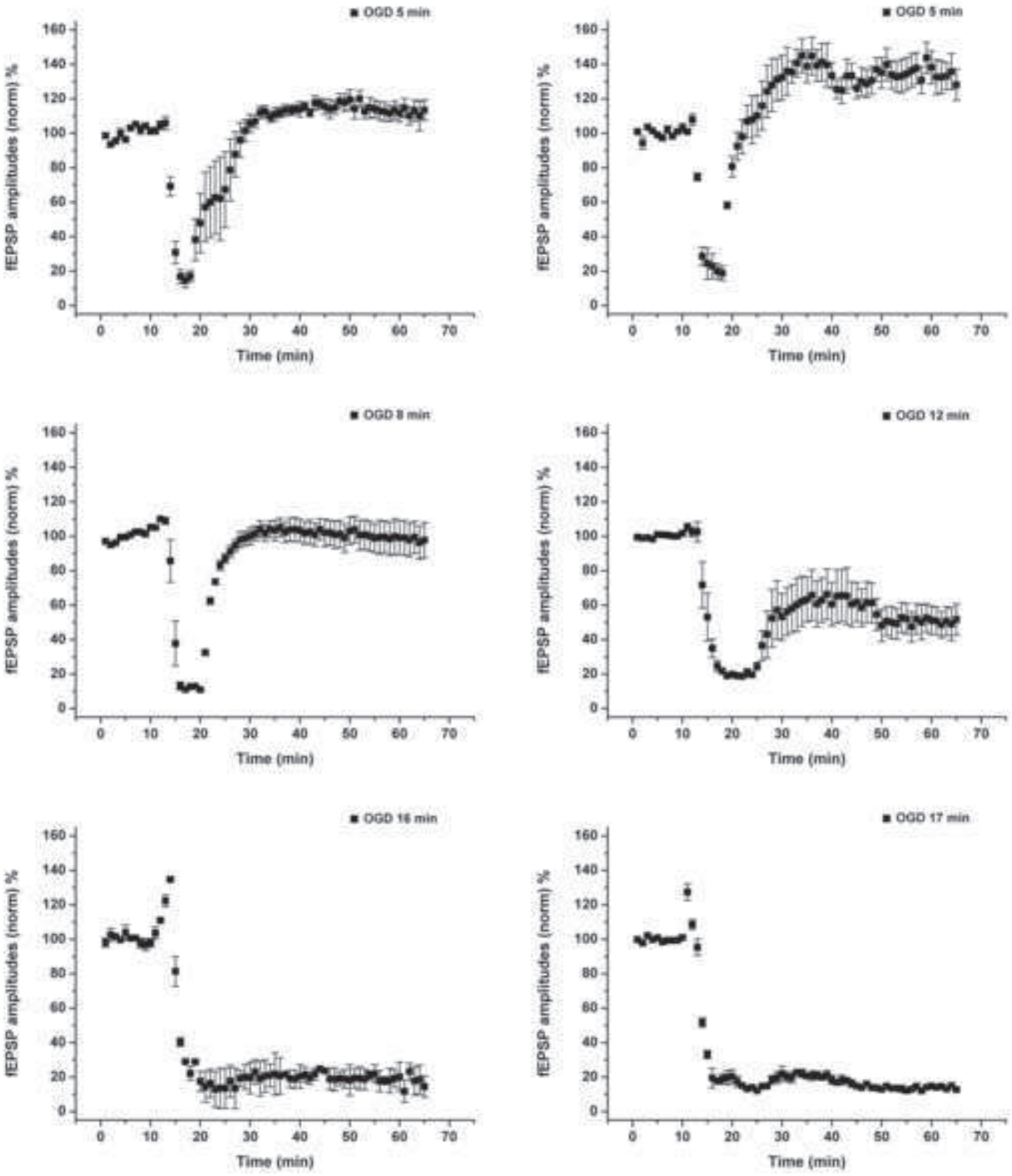

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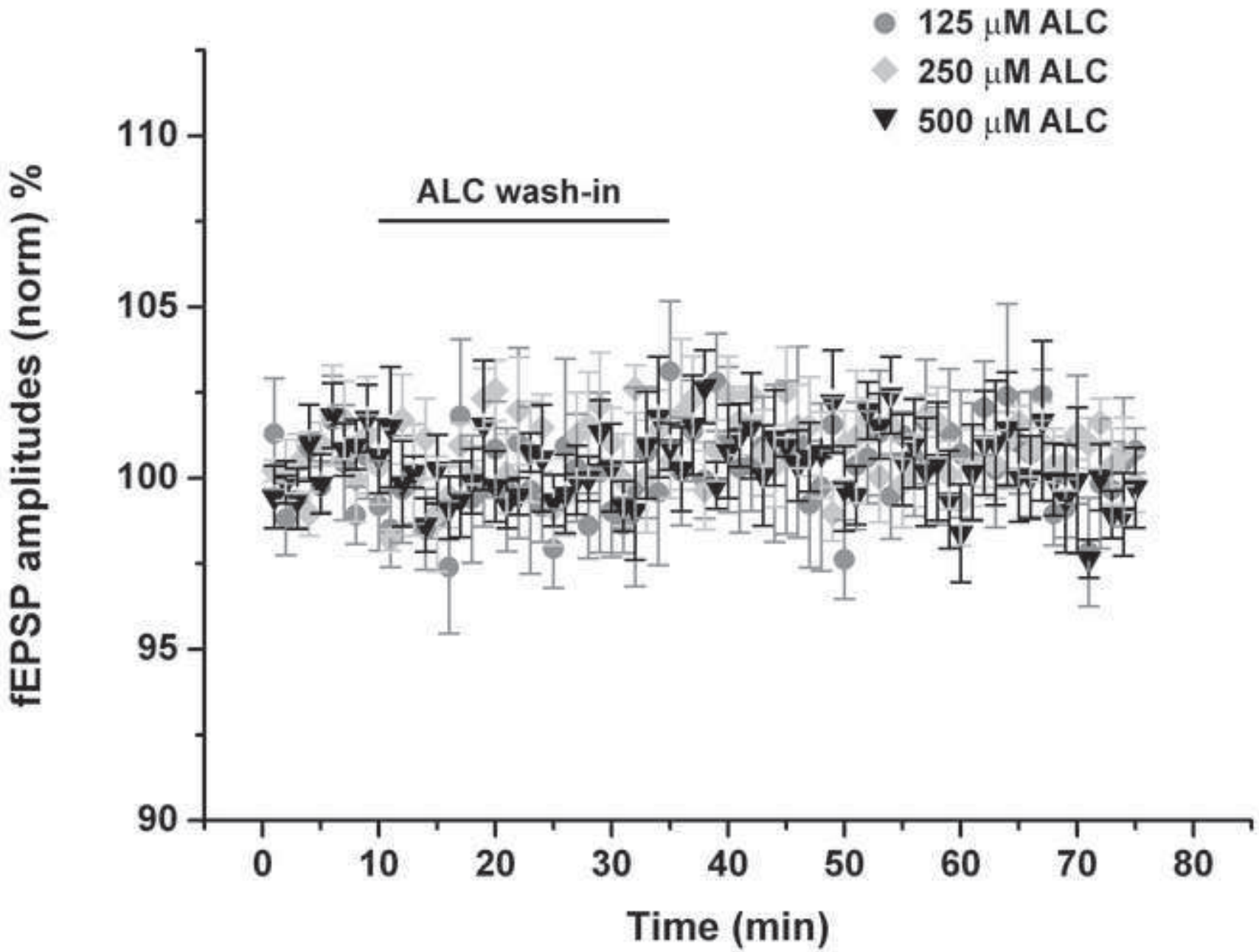

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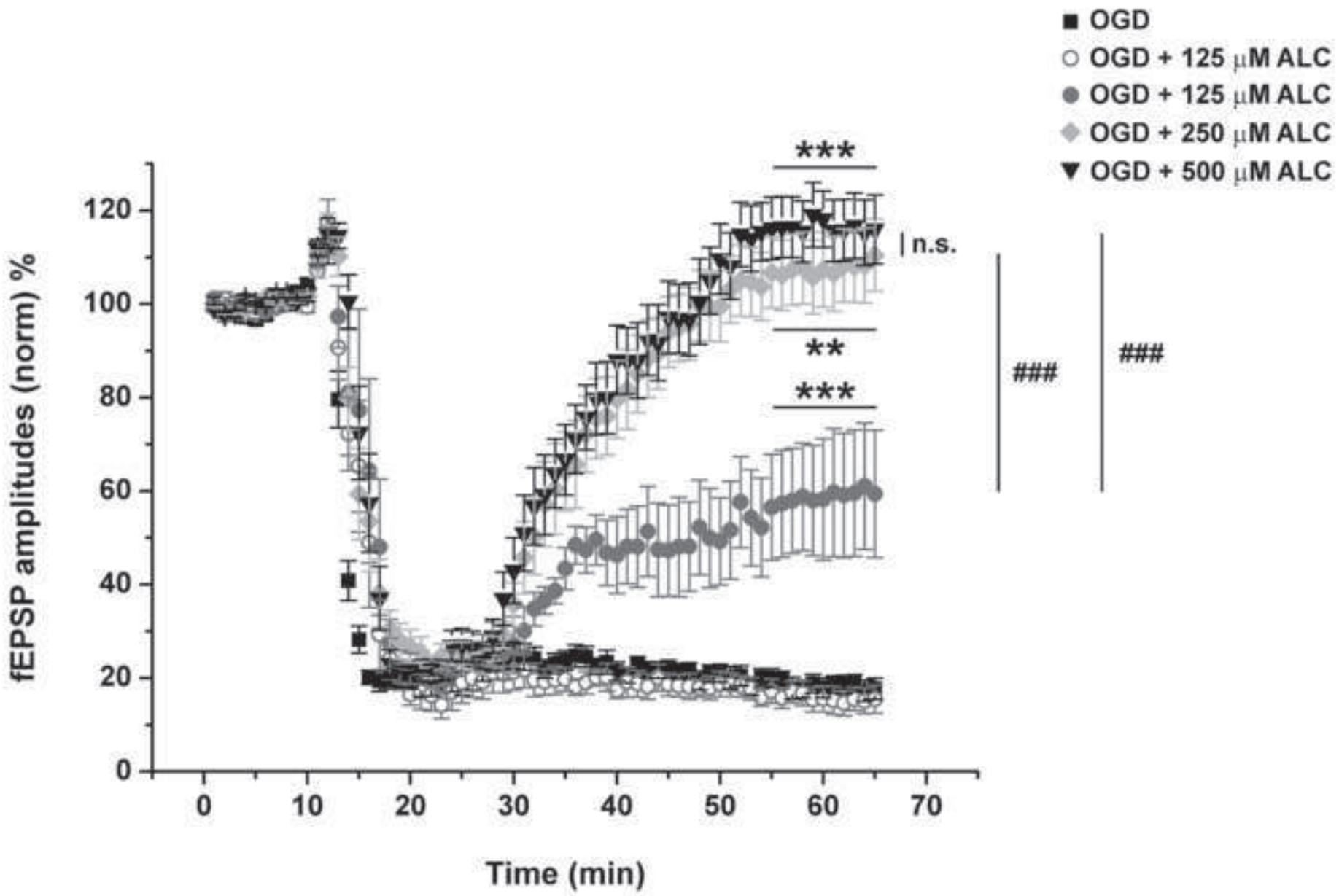

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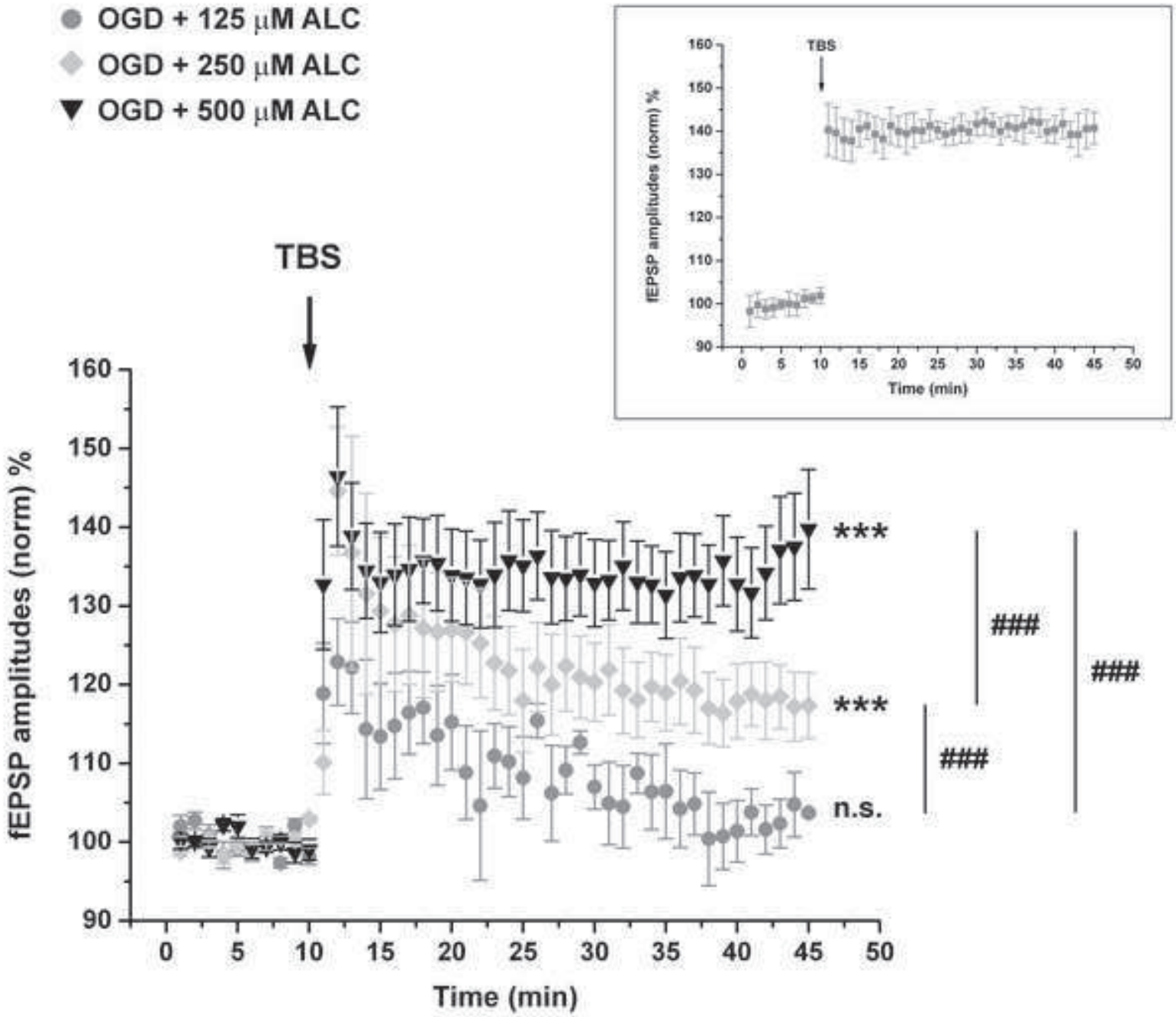

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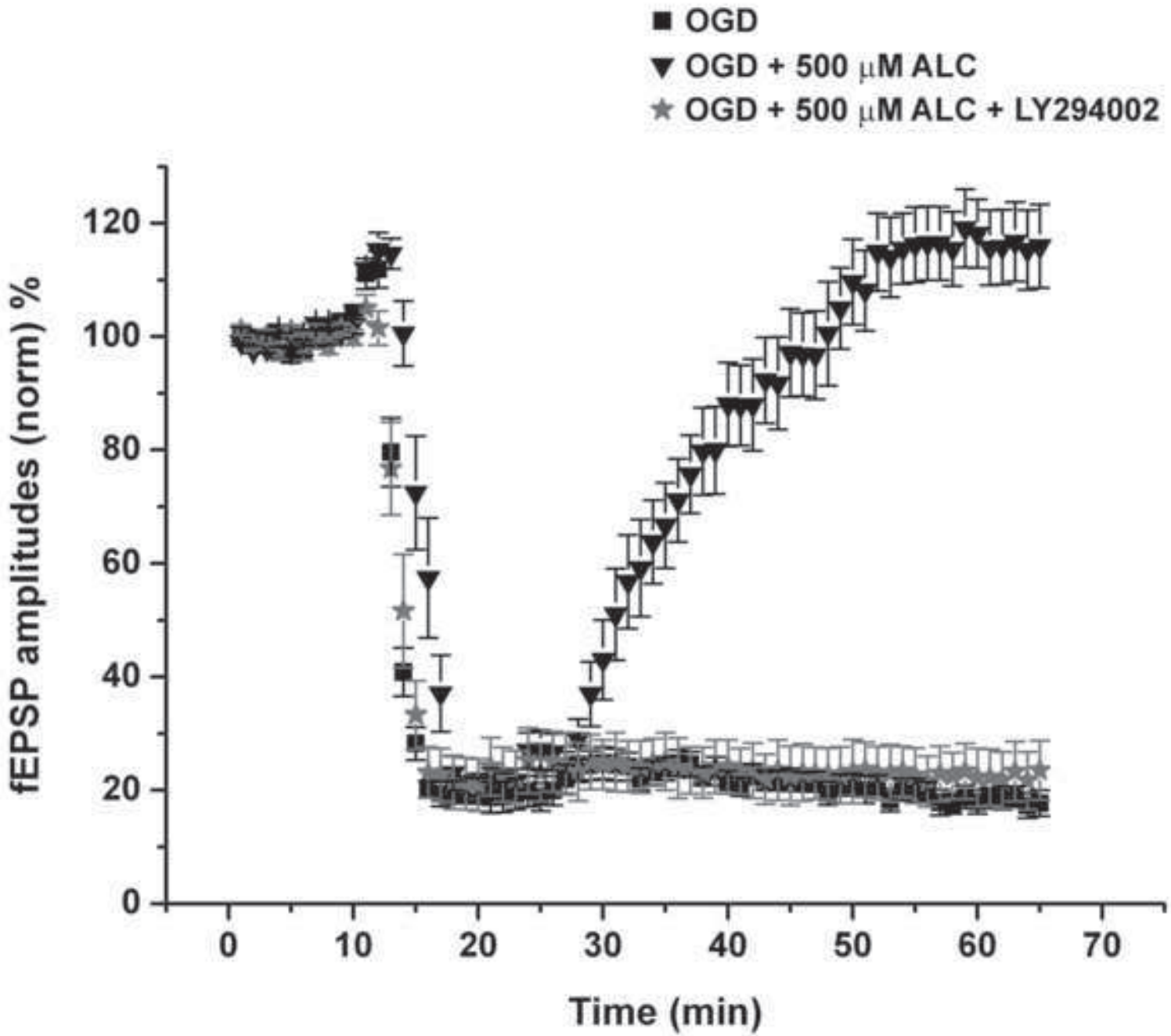

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